RXR Agonists Activate PPARα-Inducible Genes, Lower Triglycerides, and Raise HDL Levels In Vivo

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Abstract—Peroxisome proliferator–activated receptors (PPARs) and retinoid X receptors (RXRs) are members of the intracellular receptor superfamily. PPARs bind to peroxisome proliferator–response elements (PPREs) as heterodimers with RXR and as such activate gene transcription in response to activators. Fibrates like gemfibrozil are well-known PPARα activators and are used in the treatment of hyperlipidemia. We show that the RXR ligand LGD1069 (Targretin™), like gemfibrozil, can activate the PPARα/RXR signal-transduction pathway, including transactivation of the bifunctional enzyme or acyl-CoA oxidase response elements in a cotransfection assay. The activation also occurs in vivo, whereby in rats treated with LGD1069 or gemfibrozil, bifunctional enzyme and acyl-CoA oxidase RNA are induced and the combination of LGD1069 and gemfibrozil leads to a greater induction. Importantly, in hypertriglyceridemic *db/db* mice treated with AR α agoni , and the combination again has significantly greater efficacy. out changing apoA-I RNA expression. This observation suggests the use of RXR–selective agonists, " "either alone or in combination with a fibrate as a new therapeutic approach to treating patients with *Thromb Vasc Biol.* **1998;18:272-276.)**

Key Words: RXR ■ PPAR ■ rexinoids ■ hypertriglyceridemia ■ low HDL

Peroxisome proliferator–activated receptors are members of the intracellular receptor superfamily. Three subtypes have been identified, PPARα, PPARβ (NUC1 or FAAR or PPARδ), and PPARγ (see References 1 through 4 and references therein). PPARs bind to PPREs as heterodimers with the RXR and, in response to PPAR ligands,[5,6] activate gene transcription.

A diverse array of compounds, including plasticizers, fatty acids, eicosanoids, leukotrienes,[7] indomethacin,[8] and the fibrate class of lipid-lowering drugs like gemfibrozil and fenofibrate,[9] activate PPARα, while thiazolidinediones and prostaglandin J2 are PPARγ ligands.[10–12] In fibrate-treated animals, there is a rapid increase in the expression of genes that encode enzymes for the β-oxidation of fatty acids such as AOX and enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase (bifunctional enzyme).[13] PPREs have been identified in the promoters of these genes, suggesting that activation of the peroxisomal fatty acid β-oxidation pathway contributes to the lipid lowering observed with fibrates.

We have recently shown that the RXR/PPARγ heterodimer is activated by RXR agonists.[4] This finding emphasizes the permissive nature of the RXR/PPAR heterodimer, whereby either partner can bind ligand and activate gene expression. RXR agonists have similar effects as thiazolidinediones; they induce adipocyte differentiation,[14] lower elevated glucose and insulin levels, and improve insulin resistance in *ob/ob* and *db/db* mice.[15] We refer to these RXR-selective ligands as "rexinoids" because their pharmacology is clearly distinct from "retinoids," which are retinoic acid receptor activators that mimic the action of retinoic acid.[16]

We hypothesized that rexinoids would mimic the effects of fibrates via activation of the RXR side of the RXR/PPARα heterodimer. Here we demonstrate for the first time that rexinoids elicit similar responses as PPARα activators in vivo. In particular, expression of the bifunctional enzyme and AOX gene is induced in rat livers by gemfibrozil or an RXR-selective agonist LGD1069 (Targretin™) treatment.[17] The combination of LGD1069 and gemfibrozil gives a much stronger induction. Further, in *db/db* mice, RXR activators like LGD1069[17] and LG100268[18] lower triglyceride levels. HDL-C levels are also raised in rexinoid-treated mice. This finding demonstrates a convergence of the RXR and PPAR pathways in vivo and suggests that RXR modulators can be used in the treatment of lipid disorders such as hypertriglyceridemia and low HDL-C levels.

Methods

Gemfibrozil was purchased from Sigma Chemical Company, and LGD1069 and LG100268 were synthesized at Ligand Pharmaceuticals, Inc.

Plasmids and Cotransfection Assays

The bifunctional enzyme PPRE-containing reporter was constructed by ligating a 112-base pair oligonucleotide (5′-GATCC CCT TTG

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Selected Abbreviations and Acronyms

AOX = acyl-CoA oxidase
HDL-C = HDL cholesterol
PPAR = peroxisome proliferator–activated receptor
PPRE = peroxisome proliferator–response element
RXR = retinoid X receptor

ACC TAT TGA ACT ATT ACC TAC ATT AGATCC CCT TTG ACC TAT TGA ACT ATT ACC TAC ATT AGATCC CCT TTG ACC TAT TGA ACT ATT ACC TAC ATT AGATC-3') containing three copies (in bold) of the bifunctional enzyme PPRE[19] into the *Bam*HI site of pBL-tk-Luc.[20,21]

Cotransfection assays were performed as previously described.[22] Control cells received an equivalent amount of vehicle. Transfections were done in triplicate. Each point represents the mean±SD. The experiments were repeated at least twice. A representative experiment is shown.

In Vivo Studies

Male Sprague–Dawley rats (225 to 250 g) were obtained from Harlan Sprague Dawley (San Diego, Calif). Animals (four per treatment group) were fed standard laboratory diets. Animals were treated with compounds via gavage (5 mL·kg^{-1}·d^{-1}) for 7 consecutive days. The vehicle contained Tween 80, PEG 400, and carboxymethylcellulose (0.05:9.95:0.9). Liver RNA was isolated by RNAzol technique (Tel-Test Inc). The rat bifunctional enzyme cDNA and human GAPDH cDNA (Ambion) were labeled with [^{32}P]dATP by random priming. AOX cDNA (177 base pairs) was inserted into the *Pst* I and *Hind*III sites of pGEM-4 vector (Promega). Antisense AOX riboprobe was made by T7 RNA polymerase. Northern blot analysis was performed by standard techniques.

For protein extracts, livers were homogenized in 10% sucrose, 3 mmol/L imidazole (pH 7.4), and clarified by centrifugation. Protein extract (100 μg per lane) was resolved by 10% denaturing polyacrylamide gel electrophoresis and transferred to Immobilon-P transfer membrane (Amersham). The membrane was incubated with 5% powdered milk in PBS for 30 minutes at room temperature. After washing with PBST (PBS with 0.075% Tween 20), the membrane was incubated with a polyclonal anti–bifunctional enzyme antibody in PBST. The membrane was again washed with PBST, incubated with ^{125}I-labeled protein A, and the antigen-antibody complex visualized by autoradiography. The band intensities on the Northern and Western blots were quantified by a Phosphorimager (Molecular Dynamics).

Female C57BLKs/J-*m*+/+*db* mice (7 weeks old at commencement of study, nine animals per treatment group) were dosed with vehicle or gemfibrozil, LG100268, or the combination of gemfibrozil and LG100268, as indicated in the figure legends, once daily by gavage (0.6 mL/42 g) for 14 days. In a separate study, animals were dosed with vehicle (eight animals) or LGD1069 (seven animals) for 14 days. On the days indicated, animals were fasted for 3 hours and a sample of blood was drawn. The GPO-Trinder kit (Sigma) was used to measure plasma triglycerides. On day 15, the animals were sacrificed and plasma HDL-C levels measured by the phosphotungstic acid magnesium ion precipitation technique (Boehringer Mannheim).

Total liver RNA was prepared and analyzed by Northern blotting, with a ^{32}P-labeled probe specific to apoA-I. The blot was then stripped and rehybridized to a probe specific to GAPDH. The bands were quantified by a Phosphorimager (Molecular Dynamics). The intensity of the apoA-I signal in each lane was normalized to the intensity of the GAPDH signal of the same lane and the mean of each group calculated.

Results and Discussion

To study the effect of rexinoids and fibrates on the RXR/PPARα heterodimer, we used the cotransfection assay with PPARα and RXR expression vectors and a reporter containing three copies of the PPRE identified in the AOX gene[5] or the bifunctional enzyme gene. The transcriptional response increases 4-fold with gemfibrozil and 13-fold with LGD1069, an RXR-selective agonist (K_d 21–36 nM for RXRs[17]) on the AOX PPRE (Fig 1A). For the bifunctional enzyme PPRE, the transcription response was increased by 3-fold and 5-fold with gemfibrozil and LGD1069, respectively (Fig 1B). This result is consistent with our earlier data showing activation of RXR/PPARα with 9-*cis* retinoic acid (a retinoic acid receptor and RXR agonist[5,6]). However, the greatest response (49-fold with the AOX PPRE and 12-fold with the bifunctional enzyme PPRE) was observed in the presence of both gemfibrozil and LGD1069. This finding shows that a greatly enhanced transcriptional response occurs when both partners of the RXR/PPARα heterodimer are activated by their respective agonists.

Figure 1. Activation of the acyl-CoA oxidase and bifunctional enzyme (BE) PPRE by RXR and PPARα agonists. CV-1 cells were transfected with pCMVhPPARα (ref 5) and pRShRXRα (ref 23) and a reporter containing three copies of the AOX PPRE (ref 5) (A and C) or three copies of the PPRE identified in the bifunctional enzyme gene (B and D). PPARα and RXRα were used in A and B, while PPARα and RXRα(L451A) (ref 24) were used in C and D. Gemfibrozil and LGD1069 were added, to a final concentration of 100 μmol/L and 1 μmol/L, respectively.

We hypothesized that the activation of the AOX and bifunctional enzyme PPREs and the synergistic activation by PPARα and RXR ligands occur through activation of the RXR/PPARα heterodimer. If this is the case, a nonfunctional RXR is expected to blunt the transcriptional response. We used such a mutant of RXRα, RXR(L451A) (Reference 31 and Schulman et al, unpublished data, 1997). In this mutant RXRα, the amino acid at position 451 is changed to alanine. This region has been identified to be important for ligand-induced transcriptional activation by the receptor (the AF-2 domain) and is situated at the extreme carboxy- terminus of the receptor. RXR(L451A) binds RXR ligands with normal affinity but cannot activate transcription in response to ligands.[24] We show that when RXR(L451A) is partnered with PPARα, transcriptional activation in reponse to gemfibrozil or LGD1069 drops significantly (Fig 1C and 1D). In particular, activation with gemfibrozil and LGD1069 drops by 88% and 97% on the AOX and bifunctional enzyme PPREs, respec-

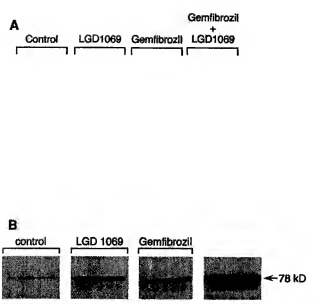

Figure 2. Induction of the bifunctional enzyme and AOX gene in rats treated with gemfibrozil and LGD1069. Rats were treated with vehicle (control), gemfibrozil (100 mg · kg^{-1} · d^{-1}), LGD1069 (30 mg · kg^{-1} · d^{-1}), or gemfibrozil plus LGD1069 (100 mg · kg^{-1} · d^{-1} and 30 mg · kg^{-1} · d^{-1}, respectively) by gavage for 7 days. A, Northern blot analysis was performed with 10 μg of total liver RNA per lane and a bifunctional enzyme (BE), AOX, or GAPDH probe. B, Western blot analysis was performed with 100 μg of protein extract from livers of treated animals and a polyclonal antibody to the rat bifunctional enzyme. The position of the expected band (78 kD) is denoted by an arrow.

tively (Fig 1; compare 1A with 1C and 1B with 1D). The simplest explanation is that the transcriptional activation of the AOX and bifunctional enzyme genes by both compounds is mediated via the PPARα/RXR heterodimer. Our interpretation is also consistent with published data demonstrating an increased transcription rate of both the AOX and bifunctional enzyme genes in response to fibrates.[25,26]

Because both gemfibrozil and LGD1069 activate the RXR/PPARα heterodimer and the bifunctional enzyme and AOX genes are induced in the livers of rats treated with fibrates,[13] we determined whether these genes are regulated by RXR agonists in vivo. Bifunctional enzyme and AOX RNA levels in livers of treated animals were determined by Northern blotting (Fig 2A). AOX RNA expression is induced 2-fold by gemfibrozil and 2.5-fold by LGD1069. In agreement with the cotransfection data, the induction is greater in the presence of both inducers (4-fold). Similarly, the bifunctional enzyme RNA was increased 4.4-fold and 4.8-fold by gemfibrozil and LGD1069 treatment, respectively (Fig 2A), and is also significantly greater in the presence of both compounds (11-fold) compared with treatment with a single agent. Hence, PPARα and RXR agonists induce hepatic expression of AOX and bifunctional enzyme RNA in treated animals.

To determine whether there was an increase in protein expression, Western blot analysis was performed with protein extracts from livers of animals similarly treated. Gemfibrozil or LGD1069 induce bifunctional enzyme protein expression (Fig 2B). A much stronger induction was observed in the presence of both compounds, consistent with both the cotransfection data and Northern blot analysis (Figs 1 and 2A). Although Western blot analysis is not the most accurate method to quantify proteins, the signal intensities indicate that the increased level of gene expression is mirrored by increased protein expression.

Clinically gemfibrozil is used for the treatment of hypertriglyceridemia, an independent risk factor for cardiovascular diseases that often correlates with low HDL-C levels. Because rexinoids activate the RXR/PPARα heterodimer and activate transcription of fibrate-responsive genes, we next determined whether RXR agonists also lower triglyceride levels in the hypertriglyceridemic *db/db* mice. These mice have a deranged leptin signaling pathway due to a mutation in the leptin receptor.[27] They are obese, hyperglycemic, and have elevated triglyceride levels that continue to increase over time in the untreated state (Fig 3A). After treatment, triglyceride levels decrease by 32% in LGD1069-treated mice compared with control mice.

We next tested a more potent and selective RXR ligand and agonist, LG100268 (K_d <5 nM[18]), for its ability to lower triglycerides, either alone or in combination with gemfibrozil. LG100268 also activates the RXR/PPARα heterodimer in a cotransfection assay (Reference 28 and data not shown). Animals treated with near-maximum effective doses of LG100268 or gemfibrozil show a 41% and 30% decrease in triglyceride levels, respectively, compared with control animals at day 11 of the study (Fig 3B). Importantly, the combination of LG100268 and gemfibrozil is significantly more efficacious in triglyceride lowering than either compound alone, decreasing triglycerides by 64% compared with control animals. Hence, the combination did not just prevent the rise in triglyceride levels observed with gemfibrozil or LG100268 alone but decreased them to the normal level seen in lean littermates.[29] No significant difference in body weight was observed in treated versus control animals.

Low HDL-C level is a risk factor for cardiovascular disease. Since elevated triglyceride levels often correlate with low HDL-C levels, we investigated whether LG100268 elevates HDL-C in these mice. LG100268 raises HDL-C levels by 27% compared with vehicle-treated animals (Table). Gemfibrozil alone did not significantly raise HDL-C levels, consistent with published data in rodents.[30]

To determine whether the rise in HDL-C levels is due to induction of apoA-I gene expression, we performed Northern blot analysis of liver RNA from treated animals. There was no change in apoA-I RNA expression in any of the treated groups compared with control animals (data not shown). Hence, the increase in HDL-C is not due to increased apoA-I gene expression and may be secondary to triglyceride reduction.

This is the first demonstration that RXR agonists mimic PPARα activators in vivo. They induce genes normally induced by fibrates, lower triglycerides, and raise HDL-C in an animal model with dyslipidemia. Further, the combination of both compounds has greater efficacy in triglyceride lowering than either compound alone.

Our results with the RXRα (AF-2) mutant indicate that the ligand-inducible transcription-activating domain of RXR contributes not only to the RXR but also to the PPARα agonist response. Similarly, preliminary experiments with PPARγ (AF-2) and RXR (AF-2) mutants indicate that

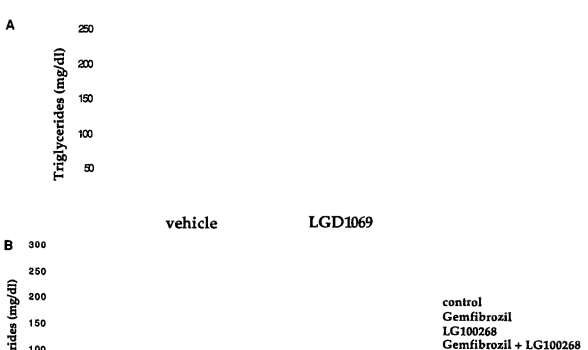

Figure 3. Triglyceride lowering by RXR agonists and PPARα/RXR combination. RXR agonists lower triglycerides, and the combination of PPARα and RXR activators has greater efficacy in triglyceride lowering than a single agent. A, *db/db* mice were treated with vehicle or LGD1069 (30 mg · kg^{-1} · d^{-1}) by gavage for 14 days. B, *db/db* mice were treated with vehicle (control), gemfibrozil (50 mg · kg^{-1} · d^{-1}), LG100268 (20 mg · kg^{-1} · d^{-1}), or a combination of gemfibrozil and LG100268 (50 mg · kg^{-1} · d^{-1} and 20 mg · kg^{-1} · d^{-1}, respectively) by gavage for 15 days. The animals were bled on the days shown and plasma triglyceride levels determined. Each data point represents the mean±SEM. *Significantly different from control group ($P<.05$) as determined by Student's *t* test. *Significantly different ($P<.05$) compared with treatment with a single agent (either gemfibrozil or LG100268).

mutating either receptor severely compromises the total activity of the heterodimer (data not shown). This observation suggests that both PPAR and RXR activating domains contribute to the total transcriptional activity induced by PPAR and RXR agonists.

PPARα and RXRs bind as a heterodimer to PPREs identified in promoters of several genes. Some of these genes are involved in lipid metabolism.[31] Further, disruption of the PPARα gene by homologous recombination prevents peroxisome proliferation and induction of target genes in response to fibrates.[32] PPARα knockout mice also have abnormal lipid metabolism. Hence, the RXR/PPARα heterodimer is the likely target for fibrates. In support of this hypothesis, clofibric acid and clofibrate induce a conformational change in PPARα[33] and ciprofibrate enhances binding of the heterodimer to DNA,[34] suggesting a direct interaction between fibrates and PPARα. Our results suggest that the RXR/PPARα heterodimer is also the target for rexinoids.

We speculate that hypoalphalipoproteinemia (low HDL-C) and hypertriglyceridemia (a risk factor for cardiovascular disease [35]) are treatable with rexinoids, or even better, with the combination of a rexinoid and a fibrate. It may be possible to use lower doses of a PPARα and RXR activator to obtain the same or increased efficacy. Combining these two classes of compounds may improve lipid profiles and have reduced side effects and toxicity. The combination may offer a novel and potentially important paradigm for the treatment of atherosclerotic cardiovascular diseases.

RXR Agonists Elevate HDL Cholesterol Levels in *db/db* Mice

Treatment	Number of Animals	HDL Cholesterol, mg/dL	SEM
Vehicle	9	112.6	3.9
Gemfibrozil	7	124.7	10
LG100268	9	143.6*	12.6
Gemfibrozil + LG100268	9	152.4*	10

db/db mice were treated with gemfibrozil, LG100268, or a combination of the two compounds as in the legend to Fig 3B and killed after 15 days. Plasma HDL-C levels were measured as described in "Methods."

*Significantly different from control animals ($P<.05$) by Student's *t* test.

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